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Order–disorder phenomena induced by *N*-ethyl maleimide in skinned frog sartorius muscle

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Yagi (1992) described the effect of *N*-ethyl maleimide (NEM) on the X-ray observed structure of chemically skinned frog skeletal muscles. Yagi's hypothesis is that this reagent is specific for the reactive SH groups of the myosin head (S1). However NEM will also interact with lysine and histidine residues (Mahler & Cordes, 1971, Table 7·4; Brewer & Riehm, 1967). Such reactions (alkylations) will change the net (negative) electrical charge on the protein, because they affect the pKs of these positively charged groups. These changes may affect the order–disorder state of the muscle fibre by charge interactions (Elliott, 1992; Elliott & Regini, 1993) rather than by the effect that Yagi postulates.

We therefore measured the net electrical charge in the A- and I-bands of skinned frog sartorius muscles at room temperature, 20 °C, following as closely as possible the procedures used by Yagi (1992) and using the microelectrode technique developed by Bartels & Elliott (1985). The results of our experiments are shown in Table 1. In the relaxed saponin-skinned frog sartorius muscle, the A-band and I-band charges are increased about twofold (by 82 %) by 1 mM NEM (as used by Yagi). We also observe a 40 % increase with NEM in the charge in the A-band in Yagi's rigor solutions.

Table 1. The A- and I- band Donnan potentials (identical in relaxed muscle) measured in Yagi's relaxing solutions, at pH 7·0 with and without 1 mM NEM, and the calculated fixed-charge concentrations for proteins (standard deviations shown, $P < 0{\cdot}001$, *t* test)

Relax	$E = -2{\cdot}0 \pm 0{\cdot}6$ mV ($n = 319$)	$[Pr^-] = 34 \pm 9$ mM
Relax + NEM	$E = -3{\cdot}7 \pm 0{\cdot}7$ mV ($n = 273$)	$[Pr^-] = 62 \pm 11$ mM

The order–disorder effects observed by Yagi, notably the disappearance of the myosin layer lines in relaxed muscle treated with NEM, may result from the disordering effect of the extra charge (Elliott, 1992). Regini & Elliott (1993) reported that the net charge could be reduced by increasing the temperature; this increases the visibility of these layer lines. NEM seems to be a rather promiscuous reagent, and one should use caution in ascribing the effects of this ligand to a specific interaction with SH groups in situations where the net electrical charge on molecules or assemblies of molecules may also be important.

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An estimate of net Ca loss from the cell during a single beat in rat ventricular myocytes

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In rat ventricular muscle the first contraction after a rest is greater than that during steady-state stimulation. This is due to a net loss of cellular calcium during stimulation because the Ca entry into the cell (via the Ca current) is less than the efflux (primarily on Na–Ca exchange). The aim of the present work was to measure both these fluxes and compare them with the Ca content of the sarcoplasmic reticulum (s.r.) as estimated electrophysiologically (Varro *et al.* 1993). $[Ca^{2+}]_i$ was measured using the fluorescent Ca indicator indo-1 in myocytes voltage-clamped with the perforated patch (Amphotericin-B) technique.

Figure 1 shows (*B*) that the magnitude of the systolic Ca transient decreases after recommencing stimulation. The membrane current record (*A*) shows that there is little effect on the calcium current. However, the inward tail current which reflects Ca extrusion by Na–Ca exchange (Fedida *et al.* 1987) decreases with stimulation. In this experiment the integral of the tail current is 7·5 pC on pulse 1 and 1·7 pC on pulse 12. Correcting for the stoichiometry of the exchange and the fraction of Ca^{2+} removed from the cell by other mechanisms, we calculate that 7·4 μmol (l cell)$^{-1}$ extra Ca is lost from the cell on the first pulse. This can be compared with the total cell s.r. Ca content of about 120 μmol (l cell)$^{-1}$ (Varro *et al.* 1993).

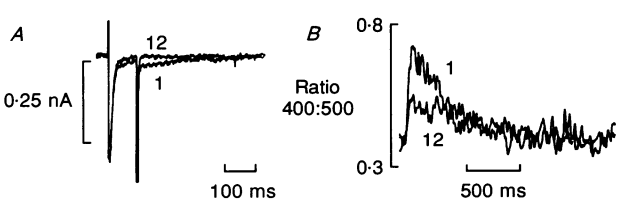

Fig. 1. Records of membrane current (*A*) and $[Ca^{2+}]_i$ (indicated by indo-1 fluorescence ratio at 400 and 500 nm) (*B*) on the first and twelfth pulse after starting stimulation following a 1 min duration rest. 100 ms duration pulses were applied at 0·5 Hz to 0 mV from −40 mV.

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Modulation of the effects of gadolinium on the contraction and intracellular calcium ($[Ca^{2+}]_i$) transient of isolated rat ventricular myocytes by NaH_2PO_4

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Gadolinium (Gd^{3+}) is known to block stretch-activated events (SAEs) in many cell types. We have investigated the possibility that Gd^{3+} has actions on single, unstretched, adult, rat ventricular myocytes.

Figure 1*A* shows that a 5 min exposure to 10 μM Gd^{3+}, the dose typically used to suppress SAEs, caused no significant reduction in the contraction ($0 \pm 1{\cdot}3$ %) nor in the $[Ca^{2+}]_i$ transient, measured with fura-2 AM ($-2 \pm 3{\cdot}1$ %) (mean ± S.E.M., $n = 6$ cells, $P > 0{\cdot}05$, two-tailed Wilcoxon matched pairs test). However, in the absence of 0·33 mM NaH_2PO_4 in the superfusing solution it was found that 10 μM Gd^{3+} now had a profound negative inotropic effect (Fig. 1*B*). There was a significant reduction, of 89 ± 3 %, in the contraction and of 82 ± 8 % in the $[Ca^{2+}]_i$ transient ($n = 9$, $P < 0{\cdot}01$).

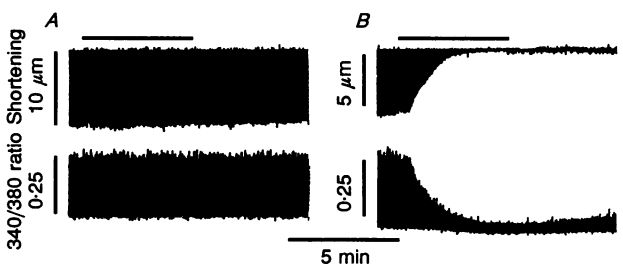

Fig. 1. The effect of 10 μM Gd^{3+} (bar) on the cell shortening (upper traces) and 340/380 nm fluorescence ratio ($[Ca^{2+}]_i$ transient, lower traces) of rat ventricular myocytes in the presence (*A*) or absence (*B*) of 0·33 mM NaH_2PO_4. Stimulation frequency was 0·5 Hz, temperature 24 °C.

We show that Gd^{3+} has actions on unstretched, adult cardiac myocytes (Fig. 1*B*). These effects are consistent with a block of L-type Ca^{2+} channels reported in other cell types. The effects of Gd^{3+} are attenuated by extracellular NaH_2PO_4 which may, with $GdCl_3$, form the sparingly soluble gadolinium orthophosphate ($GdPO_4.H_2O$) and reduce the free $[Gd^{3+}]$ (Tananaev & Petushkova, 1967).

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Creatine phosphate abolishes the inhibitory action of inorganic phosphate on sarcoplasmic reticulum function in isolated saponin-treated rat ventricular trabeculae

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During myocardial ischaemia, the intracellular concentration of inorganic phosphate (P_i) increases from about 2 to 30 mM and creatine phosphate (PCr) decreases from about 20 mM to less than 0·5 mM. We have studied the effects of P_i on the sarcoplasmic reticulum (SR) of saponin-permeabilized rat ventricular trabeculae. In the presence of 0·25 μM Ca^{2+}, rapid application of caffeine (20 mM) released Ca^{2+} from the SR which was detected using fura-2 (5 μM). The amplitude of the caffeine-induced Ca^{2+} transient was used as an assay of the Ca^{2+} content of the SR. In the absence of PCr, P_i (2–30 mM) caused a dose-dependent decrease in the Ca^{2+} content of the SR (Smith & Steele, 1992). P_i (30 mM) decreased the caffeine-induced Ca^{2+} transient by $45 \pm 3{\cdot}1$ % (mean ± S.E.M., $n = 14$).

In this study, it was found that this inhibitory action of P_i on the SR was modulated by PCr. In the presence of PCr (5–15 mM) the caffeine-induced Ca^{2+} transient was not reduced by P_i (5–30 mM). With 30 mM P_i the caffeine-induced Ca^{2+} transient increased by $7 \pm 2{\cdot}5$ % (mean ± S.E.M., $n = 14$) in the presence of 15 mM PCr. The effects of PCr were partially reversed by the creatine phosphokinase (CPK) inhibitor, dinitro-fluoro-benzene (1 mM). However, the effects of PCr were not mimicked by an alternative ATP regenerating system comprising phosphoenol pyruvate (15 mM) and pyruvate kinase (50 units ml^{-1}). These results suggest that reciprocal changes in $[P_i]$ and [PCr] that occur within the physiological range of cardiac activity (Elliot *et al.* 1994) do not exert a tonic inhibitory effect on SR function. The apparent interaction between P_i and PCr may result from a functional coupling of SR-bound CPK and the SR Ca^{2+} ATPase (Korge *et al.* 1993).

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Ca^{2+}-independent inhibition of actomyosin crossbridge cycling in cardiac myocytes by endothelial cell factors in response to hypoxia

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During cardiac hypoxia, endothelial agents (e.g. nitric oxide) mediate feedback coronary vascular dilatation (Dole, 1987). We determined whether hypoxic endothelial cells also influence myocardial contraction. Sheep cultured pulmonary artery and endocardial endothelial cells were superfused with hypoxic buffer (P_{O_2} 60–70 mmHg), and the effect of reoxygenated superfusate (P_{O_2} > 160 mmHg) tested on rat cardiac myocyte contraction (diode array) and intracellular Ca^{2+} (indo-1 fluorescence ratio). Both endocardial and vascular endothelial cell superfusate induced rapid, reversible reduction in myocyte twitch amplitude (−67·0 ± 4·9 %; mean ± S.E.M.), and decreased diastolic length (−1·5 ± 0·3 μm; both $P < 0{\cdot}001$; $n = 18$). Ca^{2+} transients were however unchanged, indicating altered myofilament properties. This effect was not attributable to known endothelial agents, nor to changes in pH_i in SNARF-loaded myocytes. Superfusate of hypoxic cells (to 1/20 dilution), but not normoxic cells, completely and reversibly inhibited the *in vitro* sliding of F-actin over rat cardiac myosin, and dramatically reduced actin-activated myosin S1 ATPase activity. Neither effect was observed with smooth muscle myosin. These data suggest a novel endothelial cell-mediated feedforward mechanism which senses hypoxia and depresses myocyte contraction, by releasing factor(s) that directly inhibit crossbridge cycling. This may explain the clinical phenomenon of myocardial hibernation, i.e., depressed contraction of viable myocardium during reduced coronary flow.

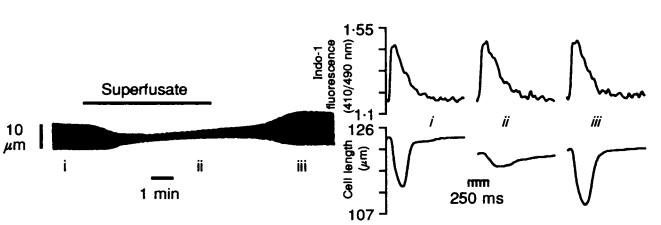

Fig. 1. Effect of hypoxic superfusate on myocyte length (left) and indo-1 fluorescence transients (right).

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Restitution of contraction and calcium current in single ventricular myocytes from guinea-pig

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At present it is not clear to what extent mechanical restitution in heart muscle depends on recovery of sarcolemmal L-type calcium current (I_{Ca}), or on recovery of intracellular calcium stores (Cooper & Fry, 1990; Lipsius *et al.* 1982). We have measured the restitution of I_{Ca} and of unloaded shortening simultaneously in single ventricular myocytes isolated by standard enzymatic techniques from guinea-pig hearts. Peak I_{Ca} was measured using the switch-clamp technique with high resistance microelectrodes (15–20 MΩ) filled with 2 M KCl. Cell length was measured using a photodiode array system. Single cells were superfused with a physiological salt solution (composition in mM: NaCl, 134; KCl, 5·4; $MgSO_4$, 1·2; Hepes, 5; glucose, 11·1; $CaCl_2$, 1·8; pH 7·4) at 35 ± 1 °C. Results are expressed as means ± S.E.M., and significance is assessed using Student's *t* test. From a basic driving frequency of 1 Hz, 200 ms test depolarizations from −45 to 0 mV were interpolated at intervals ranging from 25–30 000 ms after the preceding depolarization. Restitution of I_{Ca} is described by the sum of two exponentials with time constants of 69 ± 4 and 2500 ± 290 ms ($n = 18$). Cell shortening recovers with a single time constant (90 ± 5 ms). This is followed by a decay of developed shortening (time constant 14 500 ± 3400 ms, $n = 18$). Isoprenaline (10^{-7} M) increased the amplitude of both I_{Ca} and contraction but did not significantly alter the time course of recovery of I_{Ca}. However, the time courses of the recovery and subsequent decay of contraction are both significantly shortened (recovery time constant, 88 ± 7 ms control, 43 ± 8 ms isoprenaline, $P < 0{\cdot}001$; decay time constant 11 000 ± 2500 ms control, 1800 ± 350 ms isoprenaline, $P < 0{\cdot}004$, $n = 12$). Thapsigargin (5×10^{-7} M) reduced the amplitude of contraction by 48 % but did not significantly affect peak I_{Ca} or the time course of recovery of I_{Ca}. The time course of recovery of unloaded shortening is significantly reduced in the presence of thapsigargin (56 ± 4 ms, $P < 0{\cdot}002$). After thapsigargin the post rest decay of contraction is abolished and a slight potentiation is observed with a time course that is not significantly different from the second exponential for I_{Ca} (4300 ± 800 and 3800 ± 1300 ms respectively).

In unloaded single guinea-pig ventricular myocytes, the rate of recovery of the calcium current is faster than that of the associated cell shortening. These data suggest that mechanical restitution is not dependent solely on the rate of recovery of I_{Ca} but is also influenced by intracellular calcium stores.

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Possible effects of cADP-ribose on calcium transients and contractions in guinea-pig isolated ventricular myocytes

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Cyclic adenosine diphosphate ribose (cADP-ribose) has been suggested to play a second messenger role in the regulation of cytosolic calcium in a variety of cell types (Galione, 1993). The present experiments were designed to explore the possibility that cADP-ribose may modulate calcium-induced calcium release in cardiac cells isolated from guinea-pig ventricular muscle. Calcium-activated tail currents, recorded when action potentials were interrupted by voltage-clamp to −70 mV, were used to monitor the calcium transients (Terrar & White, 1989). Contraction was measured from the video image of cells viewed microscopically using an edge detection system. Microelectrodes were constructed from theta glass tubing to allow recording from one barrel and cytosolic application of drugs from the other.

Cytosolic infusion of cADP-ribose significantly ($P < 0{\cdot}05$, $n = 13$) depressed calcium transients measured from calcium-activated tail currents (Fig. 1*A*). The effects were dose dependent over the range 20 nM to 5 μM (concentration in the electrode dissolved in 20 mM Hepes buffer, pH 7·4). Cytosolic application of 5 μM cADP-ribose caused a corresponding reduction in the magnitude of cell shortening from $8{\cdot}7 \pm 0{\cdot}7$ to $6{\cdot}9 \pm 0{\cdot}7$ % (changes expressed as % of resting length; $n = 6$). In contrast cytosolic application of Hepes did not cause any significant change in either calcium transients (Fig. 1*B*; $n = 11$) or cell shortening ($n = 9$). The effects of 5 μM cADP-ribose on calcium transients and contraction were abolished by prior exposure of the cells to 2 μM ryanodine ($n = 6$).

These observations are consistent with the possibility that cADP-ribose may influence calcium transients in cardiac cells, perhaps by promoting calcium leakage from the sarcoplasmic reticulum during diastole.

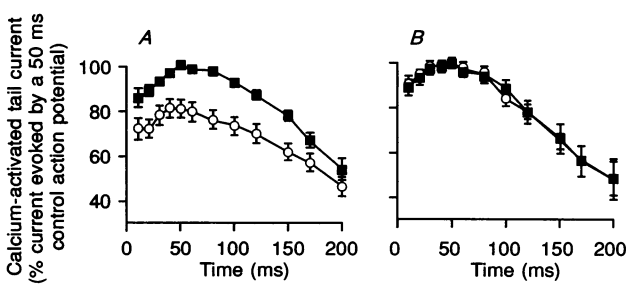

Fig. 1. Calcium-activated tail currents (± S.E.M.) before (4) and after (1) exposure to *A*, cADP-ribose (5 μM in the electrode) or *B*, Hepes (20 mM in the electrode). Temperature 36 °C.

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Inactivation of the cardiac inotrope EMD 57033 by flash photolysis in isolated rat ventricular muscle

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Increasing the sensitivity of the cardiac myofilaments to Ca^{2+} is a promising mechanism of action for new cardiac inotropic agents (Lee & Allen, 1993). EMD 57033 increases myocardial contraction predominantly by this mechanism (White *et al.* 1993). We have found that EMD 57033 is rapidly inactivated by flash photolysis. The drug produced a positive inotropic effect, associated with prolongation of relaxation and increased diastolic tension. As Fig. 1 shows, a single light flash given during the twitch caused immediate speeding of relaxation, reduced the potentiation of force (by 50 % in the muscle shown) and abolished diastolic tension. A similar fall of developed force was seen in Triton-skinned muscle preparations.

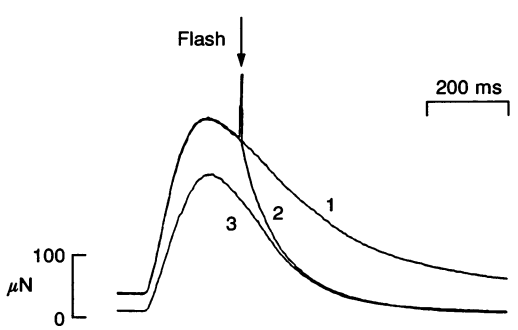

Fig. 1. The effects of a light flash given to an isolated rat ventricular trabecula in the presence of EMD 57033 (20 μM; 1 mM Ca^{2+} Tyrode solution, 0·1 Hz, 22 °C). The flash (100 mJ; 310–400 nm) was given during trace 2 of three consecutive traces.

These observations support a myofilament-based mechanism of action for the drug in intact muscle and indicate that the effects on tension and relaxation are closely interrelated. Flash inactivation may be a useful tool for studying the mechanisms of action of this compound, as previously shown with nifedipine (Morad *et al.* 1983; Gurney *et al.* 1985). Finally, these observations emphasize the need for careful controls in experiments using inotropic agents in conjunction with broad-wavelength flash photolysis of caged compounds, although laser-induced photolysis may be less prone to this effect (Simnett *et al.* 1993).

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The effect of acidosis on Ca^{2+} contractility of rat skinned myocardium and the definition of cardiac TnI role

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Acidotic pH reduces the Ca^{2+} sensitivity for skinned fibre contractility in both cardiac and skeletal fibres, but the pH effect is more pronounced in cardiac muscle. Previously, exploring the underlying mechanism for the disparity, on hamster trabeculae with skeletal TnC/cardiac TnC exchange, we gave evidence that the TnC structure itself could not modify the pH effect in cardiac muscle (Gulati & Babu, 1989). Presently, we advance the study of the mechanism of acidosis by performing TnI exchange simultaneously with TnC exchange.

Trabeculae from the rat right ventricle were used. The TnI + TnC extractions were attained by incubation with 10 mM vanadate (20 °C, 10 min incubation) per Strauss *et al.* (1992). The specimen was repleted by incubation with either sTnI + sTnC or cTnI + cTnC. The pCa–force relations were determined at pH 7 and pH 6·5, and the pCa_{50} at each pH was derived from fitted Hill plot. The unextracted controls on fast fibres from psoas muscle were also performed.

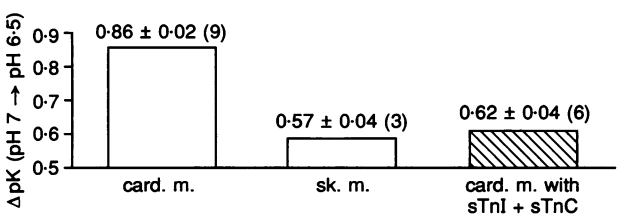

Fig. 1. The effect of pH on Ca^{2+} sensitivity in skinned myocardium. The values indicated are in pCa units ± S.E.M.

Figure 1 shows that the ΔpCa_{50} (pH 7 → 6·5) for cardiac muscle following sTnI + sTnC exchange was indistinguishable from that of skeletal muscle. Additionally, the cTnI + cTnC exchange resulted in the normal cardiac type response.

The findings firmly implicate cardiac TnI, and to a much lesser degree cTnC, in the modulation of the pH effect on Ca^{2+} contractility in the heart muscle.

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The effect of acidosis on mechanically loaded isolated rat ventricular myocytes

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The negative inotropic response of cardiac papillary muscles to acidosis depends on whether the muscle is contracting isometrically or isotonically (Lee & Allen, 1989). We have studied the contribution of mechanical loading to the negative inotropic effect of acidosis in isolated myocytes. Myocytes isolated from rat ventricles were loaded with fura-2 AM (to monitor $[Ca^{2+}]_i$) or SBFI AM (to monitor $[Na^+]_i$). Fura-2 or SBFI fluorescence, and contraction, were monitored simultaneously at 24 °C. Cells were exposed to acidosis while shortening freely, and following mechanical loading produced by attaching a carbon fibre to each end of the same cell. Mechanical loading decreased cell shortening by 63·8 ± 7·2 % (mean ± S.E.M., $n = 12$) ($P < 0·05$, paired t test).

Acidosis, produced by equilibrating a bicarbonate buffered solution with 15 % CO_2, caused a rapid decrease in contraction followed by a slow recovery. The size of the $[Ca^{2+}]_i$ transient initially decreased slightly in acidosis and then gradually increased. These changes were not significantly different in mechanically loaded and unloaded conditions. However the time course of contraction (time to peak contraction and half-time of relaxation) decreased more during acidosis in the mechanically loaded cells than in the unloaded cells, although mechanical loading had no detectable effect on the time course of the $[Ca^{2+}]_i$ transient during acidosis. In addition, the increase of $[Na^+]_i$ and diastolic $[Ca^{2+}]_i$ produced by acidosis were unaltered by mechanical loading.

In contrast to multicellular preparations, therefore, mechanical loading has little effect on the response of isolated myocytes to acidosis, although there are some differences in the time course of contraction which might be due to a different sensitivity of the contractile elements to acidosis. The difference in the present data from that obtained on multicellular preparations may be due to a number of factors, including the initial sarcomere length and the different loading conditions in the two preparations.

Carbon fibres were a gift from Professor D. Garnier, Tours. This work was supported by the BHF and the Wellcome Trust.

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Effect of extracellular pH on intracellular pH in isolated guinea-pig ventricular myocytes

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Reducing pH_o lowers pH_i in cardiac cells. We have investigated the mechanism for this. In CO_2/HCO_3-free solution, reduction of pH_o from 7·4 to 6·4 (20 mM Hepes and Pipes buffer, respectively) reversibly lowered pH_i by $0·39 \pm 0·01$ ($n = 34$; initial pH_i fall $= 0·057 \pm 0·002$ units min^{-1}; Fig. 1). A similar phenomenon was observed when using 5 % CO_2/HCO_3-buffered Tyrode solution ($n = 4$). The fall of pH_i was not due principally to inhibition of Na^+–H^+ antiport, because in the presence of 1·5 mM amiloride the rate of pH_i fall (pH_o 7·4) was slow ($dpH_i/dt = 0·015 \pm 0·001$ units min^{-1}; $n = 33$). Furthermore, the fall of pH_i in low pH_o was not blocked by amiloride ($n = 7$) or in Na_o^+-free solution ($n = 4$). The fall was not caused by H^+ influx through channels since it was unaffected by depolarization in high K_o^+ solution (115 mM; $n = 8$), was not inhibited by voltage-clamp depolarization (−70 to + 70 mV; whole cell configuration; $n = 8$), and was unaffected by putative H^+ channel blockers Cd^{2+} (5 mM; $n = 3$) and Zn^{2+} (1 mM; $n = 3$). The fall was unaffected by 0·5 mM DIDS ($n = 3$), ruling out involvement of HCO_3^- transport. In contrast, 0·2 mM 4,4′-dibenzamidostilbene-2,2′-disulphonate (DBDS), a lactate transport inhibitor (Wang *et al.* 1993), slowed the fall of pH_i by $70·4 \pm 4·1$ % ($n = 6$). The results indicate that modulation of Na^+–H^+ exchange and of HCO_3^- transport plays only a minor role in the pH_o-induced change of pH_i. The pH_i fall may be due to modulation of lactic acid transport or, alternatively, a novel DBDS-sensitive sarcolemmal acid-equivalent pathway.

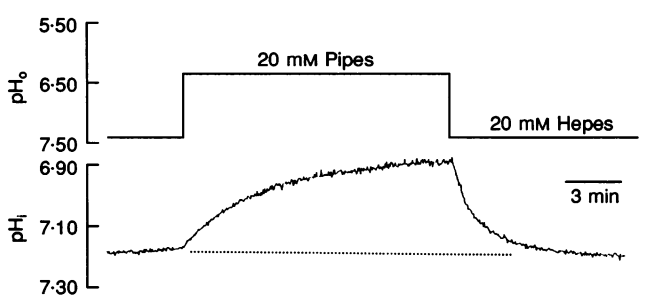

Fig. 1. Ratiometric, fluorescence recording of pH_i (SNARF-1; AM-loaded) in single myocyte perfused in CO_2/HCO_3^--free solution; temperature 37 °C.

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Cell swelling activates a chloride current in guinea-pig ventricular myocytes

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In many cell types, cell swelling activates a chloride current that contributes to regulatory volume decrease (Hoffmann & Simonsen, 1989). Using the whole-cell voltage clamp technique, we have investigated the electrophysiology and pharmacology of a swelling-activated chloride current in isolated adult guinea-pig ventricular myocytes and compared this with the isoprenaline-activated chloride current (Hume & Harvey, 1991). Cells were swollen by changing an isotonic external solution (mM: NaCl, 140; $MgCl_2$, 2; $BaCl_2$, 2; Hepes, 5; pH adjusted to 7·5 at room temperature with NaOH; Nicardipine, 2 μM and ouabain, 20 μM) to a hypotonic solution in which NaCl was reduced to 70 mM. The internal solution contained CsAsp, 110; TEACl, 20; EGTA, 10; Hepes, 5 (pH adjusted to 7·3 at room temperature with CsOH); MgATP, 10; Na_3GTP, 0·2. All experiments were performed at 36 °C.

Hypotonic swelling of myocytes was associated with activation of a time-independent outwardly rectifying current (outward rectification persisted in symmetrical internal and external chloride). The swelling-activated current was reversed by reperfusing the cell with isotonic external solution although reversal was usually incomplete. In cells under isotonic conditions, isoprenaline activated an outwardly rectifying time-independent current, which in symmetrical chloride had a linear *I–V* relationship over the range −80 to 80 mV. In cells perfused with external solution containing 78 mM chloride throughout the experiment (sucrose, 140 mM, added for iso-osmotic external solution) the reversal potential for the current activated by cell swelling was -39 ± 4 mV ($n = 5$) compared to a calculated chloride reversal potential of −35 mV. Tamoxifen (10 μM, $n = 8$), di-isothiocyanostilbene-disulphonate (100 μM, $n = 4$) and anthracene-9-carboxylic acid (1 mM, $n = 4$) inhibited the swelling-activated current by 50–70 % (as measured by the change in slope conductance at the reversal potential). None of these compounds caused significant inhibition of the isoprenaline-activated chloride current.

The electrophysiological and pharmacological characteristics of the swelling-activated current in adult guinea-pig ventricular myocytes are consistent with it being a chloride current that is distinct from that activated by β-adrenergic stimulation.

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Modulation of the hyperpolarization-activated current (i_f) by adenosine in rabbit sinoatrial myocytes

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Adenosine is an important factor in local modulation of neural control of cardiac function. In cardiac tissues, stimulation of adenosine A1-receptors and muscarinic M2-receptors have similar effects, possibly mediated by common intracellular pathways.

In rabbit SA node myocytes, adenosine has been reported to affect the hyperpolarization activated current i_f (Belardinelli *et al.* 1988) only after α-adrenergic stimulation. This is in contrast with the inhibition of i_f in the SA node by acetylcholine alone (DiFrancesco & Tromba, 1988), and would imply distinct intracellular pathways for A1 and M2 receptors. We have then set out to re-evaluate the modulation of i_f by adenosine in rabbit SA node myocytes.

Isolated myocytes were superfused at 36–36·5 °C with modified Tyrode solution containing 1 mM $BaCl_2$ and 2 mM $MnCl_2$ to block K^+ and Ca^{2+} currents. Under whole-cell voltage-clamp conditions, i_f was activated by hyperpolarizing steps, from a holding potential of −35 mV, applied at 3 s intervals.

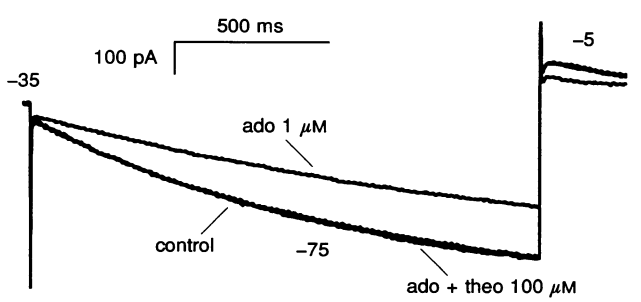

Fig. 1. Modulation of i_f by 1 μM adenosine (ado) alone and in the presence of 100 μM theophylline (theo).

As shown in Fig. 1, 1 μM adenosine (ado) reduced i_f and slowed its activation. This effect was reversible and almost fully antagonized by 100 μM theophylline (theo), an adenosine receptor antagonist. Theophylline alone induced a slight increase in i_f, probably due to phosphodiesterase inhibition. In five cells, 1 μM adenosine reduced i_f by $33{\cdot}1 \pm 5{\cdot}7\%$ ($P < 0{\cdot}05$). By applying a suitable pulse protocol, we have also shown that adenosine reduces i_f at mid-activation potentials, without changing its fully activated value ($n = 4$).

Our data indicate that, in contrast with previous reports, adenosine inhibits i_f in the absence of concomitant adrenergic stimulation by shifting the i_f activation curve. Therefore, the action of adenosine on i_f in SA node myocytes is qualitatively similar to that of acetylcholine (DiFrancesco *et al.* 1988)

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Properties of L-type calcium current in atrioventricular myocytes isolated from the rabbit heart

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In the multicellular atrioventricular node (AVN) the action potential can be abolished by manganese (Mn) (Zipes & Mendez, 1973), whilst the AVN action potential upstroke is unaffected by 10 μM TTX (Kokubun *et al.* 1982). Little is known about the role of L-type calcium current (I_{Ca}) in generating spontaneous activity in the AVN. We have examined the role of I_{Ca} in rod-shaped, Ca-tolerant myocytes from the AVN of the rabbit heart, prepared using the method described by Hancox *et al.* (1993).

Experiments were performed at 35–37 °C using the whole cell patch clamp technique. Current-clamp recordings showed that spontaneous action potentials in AVN cells were reversibly abolished by addition of nifedipine (20 μM) or cadmium (Cd; 100–200 μM) to the perfusate. Whole cell voltage-clamp recordings were made using a caesium-containing pipette solution. Depolarizing voltage-clamps were applied from a holding potential of −40 mV: a rapidly activating inward current was observed with steps more positive than −30 mV, peaking at + 10 mV in most cells and exhibiting a 'bell-shaped' current–voltage (I–V) relation. This current was blocked by nifedipine (2–20 μM) and was also sensitive to divalent cations (Cd, 100–200 μM and Mn, 1 mM). At + 10 mV the mean time-to-peak I_{Ca} was 3·3 ms (S.E.M. 0·15; $n = 12$) and the mean I_{Ca} current density was $9{\cdot}3 \pm 1{\cdot}2$ pA pF^{-1} ($n = 9$). Steady-state activation and inactivation curves were constructed, assuming a Boltzmann distribution of channel charge. Half-maximal activation occurred at −3·6 mV (slope factor $k = 6{\cdot}6$) and half-maximal inactivation at −25·8 mV ($k = 6{\cdot}5$). Using the activation/inactivation constants obtained experimentally we calculated the steady-state 'window' I_{Ca} between −70 and + 40 mV. This steady-state I_{Ca} window extended into the pacemaker potential range. Therefore, in addition to generating the action potential upstroke, it is possible that I_{Ca} may play a role in generating the pacemaker depolarization in spontaneously active AVN cells.

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Carrier-mediated lactic acid transport into isolated rat and guinea-pig cardiac myocytes detected by measuring changes of intracellular pH

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Lactic acid is transported across the heart cell membrane by a specific carrier mechanism (Poole & Halestrap, 1993; Wang *et al.* 1993). Using BCECF to measure changes in intracellular pH (pH_i) associated with lactate uptake, we have characterized the kinetics of lactate transport into single cardiac myocytes.

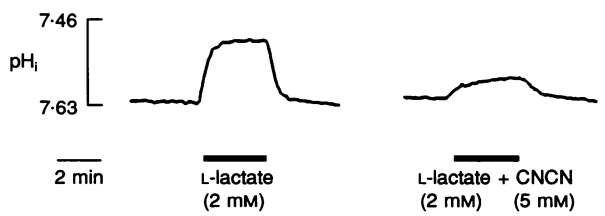

Fig. 1. pH_i changes in a rat cardiac myocyte with 2 mM L-lactate alone (left) and in the presence of 5 mM CNCN (right); temperature 23 °C.

The decrease in initial rate of pH_i change with CNCN reflects inhibition of carrier-mediated transport. Using the CNCN-inhibitable component to obtain carrier kinetics, we find that rat myocytes have a K_m for L-lactate of $2{\cdot}16 \pm 0{\cdot}21$ mM whilst guinea-pig myocytes have a K_m of $2{\cdot}2 \pm 0{\cdot}3$ mM. However, two isoforms of the lactate carrier appear to co-exist in a single myocyte, since DBDS (4,4′-dibenzamidostilbene-2,2′-disulphonate) only partially inhibits lactate transport. DBDS inhibits only 70 and 44 % of transport in guinea-pig and rat cells respectively, in agreement with previous findings for cell suspensions (Wang *et al.* 1993).

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Degradation of endothelin-1 in rat myocardium

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Raised plasma levels of endothelin-1 (ET-1) have been reported in patients with myocardial ischaemic conditions including infarction (Stewart *et al.* 1991) and angina (Toyo-oka *et al.* 1991). We have recently reported that infusion of ET-1 in anaesthetized rats causes increased incidence of arrhythmias under conditions of myocardial ischaemia (Garjani *et al.* 1993) This study investigates the nature of ET-1 destruction in rat myocardium and compares it with that in the lung.

Rats were killed and the ventricular myocardium and lungs were removed and homogenized ($0{\cdot}1$ g ml^{-1}) in phosphate buffer (0·01 M, pH 7·2, 0·1 % Triton X-100). Degradation of ET-1 (20 $\mu g\ ml^{-1}$) was assessed by incubating (37 °C, 1 h) with tissue extracts, boiling, centrifuging and drying the supernatant under nitrogen. The ET-1 was measured using reverse phase HPLC (C18, acetonitrile 15–70 %, 0·09 % TFA). Group differences were compared using the Mann–Whitney *U* test.

ET-1 was degraded by both ventricular and lung extracts optimally at acid pH. At pH 5·2, the myocardial extracts contained significantly less ET-1 degrading activity than those from the lung (58·8 %, $P < 0{\cdot}01$, $n = 5$). The degrading activity was not inhibited by EDTA (50 mM) indicating that it was not metal dependent. Degradation of ET-1 by the lung extracts produced four metabolite peaks on HPLC. Incubation of ET-1 with myocardial extracts produced only two detectable metabolite peaks, one of which co-eluted with tryptophan. Whether the second metabolite is ET-1 minus the C-terminal tryptophan remains to be confirmed. Tryptophan was also one of the four metabolites produced by lung.

At pH 6·2, the myocardial ET-1 degrading activity was greatly increased to 222 % of that at the more physiological pH 7·2 ($P < 0{\cdot}02$). Khandoudi *et al.* (1990) observed that myocardial tissue pH falls as low as pH 6·26 in normal rats and pH 6·03 in diabetic animals after 10 min myocardial ischaemia. The presence in myocardium of an acid optimum, ET-1 metabolizing enzyme which cleaves the C-terminal tryptophan is thus potentially of considerable pathophysiological significance.

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Developmental changes in calcium handling by the neonatal rat heart

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There are profound differences in the mechanical activities of neonatal hearts and adult hearts (Nakanishi & Jarmakani, 1984) and also between hypertensive and normotensive hearts (Conrad *et al.* 1991). In both cases these

differences are believed to relate mainly to alterations in the handling of calcium (Ca^{2+}) by the myocardium. When the sarcoplasmic reticulum (SR) is functionally immature, the relative contribution of the sarcolemmal sodium/calcium exchanger (Na/Ca Ex) to Ca^{2+} metabolism may be greater (Vetter & Will, 1986). Calcium uptake into the SR is inhibited by the SR membrane protein phospholambam (PLB) when this protein is unphosphorylated. This inhibition is relieved by the phosphorylation of PLB and this might also be subject to developmental variation. To test these hypotheses we have investigated oxalate-supported Ca^{2+} uptake (CaT) into SR vesicles derived from whole ventricular homogenates and Na/Ca Ex in myofibrillar-free cardiac membrane preparations in neonatal spontaneously hypertensive rats (SHR) and their Wistar–Kyoto (WKY) normotensive counterparts over a 40 day period post-partum.

In our populations, there occurred a more than 10-fold increase in ventricular weight and a 20-fold increase in body weight between postnatal days 1 and 40 in both rat strains. By contrast, ratio of ventricular weight to body weight (relative heart weight) was lower at day 40 in both populations than at day 1. These changes were paralleled by an approximate doubling in total ventricular protein content from day 1 to day 40, indicating that the synthesis of cardiac proteins is extremely high in this period. CaT into SR vesicles increased with age from day 1 to a maximum at day 12 in both strains from $0{\cdot}252 \pm 0{\cdot}02$ to $1{\cdot}082 \pm 0{\cdot}12$ nmol Ca^{2+} (mg wet wt)$^{-1}$ min^{-1} for SHR ($n = 8$ and 5 respectively, $P < 0{\cdot}0001$; mean ± S.E.M., Student's t test) and from $0{\cdot}117 \pm 0{\cdot}01$ to $0{\cdot}866 \pm 0{\cdot}04$ nmol Ca^{2+} (mg wet wt)$^{-1}$ min^{-1} for WKY ($n = 6$; $P < 0{\cdot}0001$). In addition, the PLB data showed that ^{32}P incorporation into PLB, at the protein kinase A specific site, also increases from day 1 to a maximum at day 12 in both strains. For SHR the increase was from $1{\cdot}614 \pm 0{\cdot}24$ to $2{\cdot}752 \pm 0{\cdot}29$ pmol ^{32}P (g wet wt)$^{-1}$ ($n = 6$) and from $1{\cdot}339 \pm 0{\cdot}10$ to $3{\cdot}270 \pm 0{\cdot}23$ pmol ^{32}P (g wet wt)$^{-1}$ for WKY ($n = 5$ and 6 respectively). These changes were mirrored by a decrease in Na/Ca Ex from day 1 to day 12 of $0{\cdot}843 \pm 0{\cdot}15$ ($n = 10$) to $0{\cdot}159 \pm 0{\cdot}05$ ($n = 10$) nmol Ca^{2+} (mg protein)$^{-1}$ (2 s)$^{-1}$ (SHR, WKY and Wistar data pooled; $P < 0{\cdot}0001$).

The results show a co-ordinate relationship between the amount of PLB present in the developing heart and the activity of the Ca^{2+} uptake mechanism up to day 12 in rats. Secondly there is an inverse relationship between the activities of the CaT and Na/Ca Ex with ontogenic development.

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Cyclopiazonic acid inhibits the lusitropic action of isoprenaline in isolated rabbit papillary muscles

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In cardiac muscle, β-adrenoceptor agonists such as isoprenaline (ISO) both potentiate and shorten the twitch. The precise mechanism of the latter, 'lusitropic', action is unclear, but it could be due to protein phosphorylation causing (i) an increased rate of Ca^{2+} sequestration into the SR, (ii) a faster loss of Ca^{2+} from troponin C, or (iii) a faster detachment of cross-bridges. We have tried to delineate the role of the SR in the lusitropic and inotropic effects of ISO by using cyclopiazonic acid (CPA) to selectively inhibit the SR Ca^{2+} uptake pump (Seidler *et al.* 1989).

Papillary muscles, isolated from rabbit right ventricle, were bathed in Tyrode solution in a muscle bath. In eleven control muscles (e.g. Fig. 1*A*), 1 μM ISO shortened the time to peak tension (TPT) from 219 ± 9 ms (mean ± S.E.M.) to 164 ± 3 ms. In a different set of six muscles, the addition of CPA (30 μM) by itself produced little change (increase of $6{\cdot}9 \pm 4{\cdot}2$ %) in TPT, but abolished the action of ISO to shorten the twitch (e.g. Fig. 1*B*): in fact, ISO then increased TPT significantly from 218 ± 7 to 240 ± 9 ms ($P < 0{\cdot}05$, paired t test). The relative inotropic effect of ISO was not significantly different in the absence (612 ± 83 % increase) and the presence (674 ± 114 % increase) of CPA ($P > 0{\cdot}05$, Student's t test). We conclude that in rabbit ventricle the SR plays a dominant role in the lusitropic action of ISO, but contributes relatively little to the inotropic action.

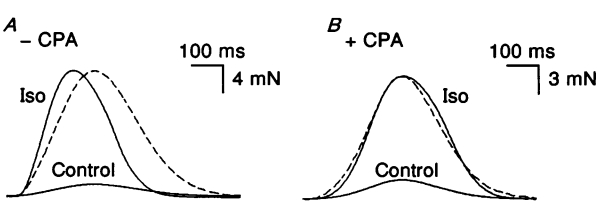

Fig. 1. Representative traces showing the effects of ISO (1 μM) on the twitch in two papillary muscles: (*A*) with no CPA; (*B*) after > 1 h in CPA (30 μM). The traces start at the time of the stimulus. The dashed line is the control trace normalized to that for ISO. 2 mM Ca^{2+}, 30 °C, stimulus rate 0·33 Hz.

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The effect of toxins from the jellyfish *Chironex fleckeri* on cardiac muscle isolated from rat and ferret hearts

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Severe disabilities and occasional deaths have been reported following poisoning by the jellyfish *Chironex fleckeri*. Injection of the isolated toxin to anaesthetized animals results in signs of cardiac toxicity characterized by the development of a fast and irregular cardiac rhythm and conduction block (Freeman & Turner, 1969). The aim of the present study was to investigate the mechanisms underlying the toxicity.

We monitored force, intracellular $[Ca^{2+}]$ ($Ca^{2+}{}_i$; using the photoprotein aequorin) and membrane potential in ferret papillary muscles, and cell shortening and $Ca^{2+}{}_i$ (using fura-2) in rat ventricular myocytes. *C. fleckeri* toxin (CFx), isolated from specimens caught off the coast of N. Queensland, Australia, was applied to these preparations at concentrations between 10–100 μg protein ml^{-1}.

In the ferret papillary muscle, CFx decreased developed force to 14 ± 3 % of control after 120 s (mean ± S.E.M., $n = 13$, $P < 0.01$); this was preceeded by a small increase in developed force at low doses of the toxin. The decrease of force was associated with a decrease in action potential duration and resting potential. These changes were followed by an increase in stimulation voltage threshold (so that some preparations became inexcitable), an oscillatory increase of $Ca^{2+}{}_i$, spontaneous depolarizations of the cell membrane, an increased in resting force and the development of spontaneous action potentials and contractions (which appeared in both stimulated and unstimulated preparations). Similar changes of contractility and $Ca^{2+}{}_i$ were observed in rat myocytes.

These observations suggest that the cardiac toxicity of CFx is due to the development of marked Ca^{2+} overload and its functional sequelae. It is unlikely that this overload is produced by non-specific damage, since the preparations can recover fully after a brief exposure of the toxin. It is unlikely to be due to catecholamine release from nerve terminals within the papillary muscles, since it is unaffected by propranolol (1 μmol l^{-1}) and was observed in single cells, nor it is likely to be due to influx via the L-type Ca^{2+} channel, since it is unaffected by nifedipine (10 μmol l^{-1}).

We thank M.T. Eldila for isolation of the toxin.

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Post-ischaemic flow debt repayment in isolated hypertrophied guinea-pig hearts is reduced because of an impaired endogenous adenosine response

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In cardiac hypertrophy the hyperaemic response following an occlusion of flow is significantly impaired (Isoyama *et al.* 1989). This may be explained by increased extravascular compressive forces (O'Gorman *et al.* 1991) or reduced vascular proliferation (Breisch *et al.* 1986). Adenosine fulfils most of the criteria for mediator of coronary blood flow and in this study we investigated the possibility that the response to endogenous adenosine is impaired in left ventricular hypertrophy.

Post-ischaemic flow debt repayment was studied in isolated perfused hearts from guinea-pigs 6 weeks after aortic constriction (AC) or sham operation. Heart weight/body weight ratio was increased with AC (0.39 ± 0.03 *vs.* 0.28 ± 0.01 %, means ± S.E.M., $P < 0.01$). In isolated buffer perfused hearts, after a 45 s occlusion of flow, hyperaemia was impaired in hypertrophy compared with sham controls in both magnitude (7.6 ± 0.45 *vs.* 9.49 ± 0.51 ml min^{-1} g^{-1}, $P < 0.05$) and duration (54.2 ± 8.94 *vs.* 75.54 ± 7.97 s, $P = 0.09$). This led to a reduced flow debt repayment (27.45 ± 4.51 *vs.* 45.77 ± 3.62 %, $P < 0.01$). The adenosine receptor antagonist 8-phenyl-theophylline (8-PT, 10^{-5} M) reduced the peak hyperaemic flow in sham (4.36 ± 0.55 *vs.* 8.81 ± 0.77 ml min^{-1} g^{-1}, $P < 0.001$) and in hypertrophied (4.02 ± 0.45 *vs.* 7.2 ± 0.6 ml min^{-1} g^{-1}, $P < 0.001$) hearts. 8-PT also reduced the duration but to a greater degree in sham (29.11 ± 3.44 *vs.* 96.67 ± 13.07 s, $P < 0.001$) than in hypertrophied (26.5 ± 6.78 *vs.* 54.63 ± 7.29 s, $P < 0.05$) hearts. Post-ischaemic flow debt repayment was also reduced more by 8-PT in sham (11.54 ± 1.48 *vs.* 59.75 ± 7.63 %, $P < 0.001$) than in hypertrophied (7.86 ± 2.49 *vs.* 30.59 ± 6.16 %, $P < 0.01$) hearts.

The greater inhibition of the hyperaemic response with 8-PT in sham control hearts suggests that release of or response to endogenous adenosine may be impaired in hypertrophy, contributing to the reduced flow debt repayment following ischaemia.

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